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Evaluation of potassium channel blockers, 4-aminpyridine and 4-aminpyridine-3-methanol, in combination with electrical neuromodulation for functional upper limb recovery following incomplete cervical spinal cord injury

Author

Juan-Sing, Czarina Catherin

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A thesis submitted in partial satisfaction

of the requirements for the degree of Master of Science

in Physiological Science

by

Czarina Catherine Juan-Sing

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ABSTRACT OF THE THESIS

Evaluation of potassium channel blockers, 4-aminpyridine and 4-aminpyridine-3-methanol, in combination with electrical neuromodulation for functional upper limb recovery following incomplete cervical spinal cord injury

by

Czarina Catherine Juan-Sing

Master of Science in Physiological Science

University of California, Los Angeles, 2020

Professor Victor R. Edgerton, Chair

Cervical spinal cord injury (SCI) is a traumatic condition where individuals lose crucial motor and sensory functions in their hands and arms. These deficits can be caused by myelin damage, which exposes juxtaparanodal potassium channels leading to large potassium effluxes and ultimately, shortened and decreased conduction. However, potassium channel blockers such as 4-aminopyridine (4-AP) and the novel derivative, 4-aminopyridine-3-methanol (4-AP-3-MeOH) prevent potassium ion leakage and increase conduction to improve motor function. Currently, it is possible to regain voluntary motor control and strength in the forelimbs and hands by using complementary electrical stimulation, pharmacological agents and training to transform dormant spinal circuitry

into active states. Here, we evaluated the extent to which potassium channel blockers, 4-AP and 4-AP-3-MeOH enable functional forelimb ability when used in combination with spinal stimulation and motor training after incomplete cervical SCI. 4-AP restored reaching and grasping function and muscle activation in rats after two weeks of treatment. 4-AP-3-MeOH, however, did not lead to improve motor functioning or muscle activation. Both drugs decreased muscle activation latency and allowed for earlier muscle activation. Our findings suggest 4-AP synergistically works with spinal stimulation and training to enable spared circuitry following SCI and can be used acutely to restore forelimb function.

The thesis of Czarina Catherine Juan-Sing is approved.

Ricardo Olcese

Fernando Gomez-Pinilla

Victor R. Edgerton, Committee Chair

University of California, Los Angeles

2020

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INTRODUCTION

Overview of spinal cord injury

Spinal cord injury (SCI) is a traumatic disease that affects roughly 250,000 individuals every year and can result in debilitating loss of motor and sensory functions (Gerasimenko et al., 2008) An estimated 1 million individuals are currently motor impaired due to SCI and, tragically, most are below the age of 30. Following traumatic cervical SCI, individuals immediately lose the functionality in their arms and hands and are unable to perform daily basic activities of living. This is reportedly the most debilitating SCI impairment and remains the highest priority target for tetraplegics (Anderson, 2004). However, while maximizing arm and hand function are of utmost significance, these individuals also suffer from overall physiological dysfunction including autonomic dysreflexia, sexual dysfunction and loss of bladder control (Aikman et al., 2018; Inskip et al., 2018; Sharif & Hou, 2017). Given their younger demographic, these individuals can face a lifetime of paralysis, continuous dependence on caregivers, poor mental health, and disruption of life goals and personal relationships. Therefore, it is crucial to investigate and rapidly implement SCI therapies that target arm and hand function to increase independence, motor ability and quality-of-life in these patients.

Current pharmacological and electrical stimulation treatments following SCI

SCI motor deficits are caused by damaged and demyelinated spinal circuitries that can no longer optimally transmit nerve impulses. However, recent studies have shown that electrically-enabled motor control (eEMC) of spinal cord can restore voluntary control in adult humans with clinically motor-complete SCI

eEMC increases the excitability of spared spinal circuitries to enable functional recovery. Studies have demonstrated that it is possible to recover full weight-bearing bipedal stepping when complementary pharmacological, electrical and physical interventions are used (Courtine et al., 2009; Duru et al., 2015; Ichiyama et al., 2008; Musienko et al., 2011). Combination therapy, which simultaneously implement eEMC, repeated motor task training and complementary pharmacological agents, enable spared motor circuits and lead to significant motor recovery that would not have been achieved using only one mode of treatment.

Spinal stimulation has similarly been shown to be effective for the restoration of upper arm and hand function after injury (Alam et al., 2018; Gad et al., 2018; Terson de Paleville et al., 2019). Cervical intraspinal stimulation elicits motor responses in forelimb muscles (Sunshine et al., 2013) and elicit reaching and grasping movements in non-injured monkeys (Zimmermann & Jackson, 2014) and spinalized rats (Alam et al., 2015). Additionally, acute stimulation of the cervical SCI improves forelimb grip strength and reaching and grasping ability (Alam et al., 2018) in rats and improves functional motor recovery in human patients (Gad et al., 2018). These results show promising treatments to restore arm and hand function - the highest priority of individuals with a cervical SCI. However, it has not been determined whether eEMC and motor training in combination with pharmacology can activate spinal networks and lead to improved functional upper motor function.

Potassium ion channel blockers as a pharmacological agent

Potassium ion channels are a crucial target in SCI treatment as myelin damage exposes juxtaparanodal potassium channels. Ion leakage from these channels increases potassium ion efflux, diminishes depolarization and action potential propagation at the nodes of Ranvier, which results in shortened or decreased amplitudes. Potassium channel blockers such as 4-aminopyridine (4-AP) enhance conduction to allow for functional motor recovery.

4-AP has been used in SCI patients to enable muscle responsivity but has not been combined with other treatments (Wiener et al., 2018). More so, 4-AP is widely used to improve functionality in lower limbs, but few studies have focused on the potential role of 4-AP in the recovery hands and arms. In an *in vitro* rat spinal cord model, low concentrations of 4-AP generated fictive locomotor patterns from ventral roots of the spinal cord (Taccola & Nistri, 2005) . Additionally, low doses of fampridine, significantly improved spasticity in patients with incomplete SCI as measured by changes in Ashworth scores (Cardenas et al., 2007). More so, 4-AP and spinal stimulation in SCI rodent models was sufficient to increase muscle responsivity in upper limbs compared to non-treated groups (Sindhurakar et al., 2017) Therefore, the mechanism of action of 4-AP makes it an attractive pharmacological agent to enable spinal excitability in upper limbs following injury.

While 4-AP is FDA-approved for gait improvement in neurodegenerative disorders (Ampyra, Acorda Therapeutics, Inc.), the drug is noted for its narrow therapeutic

potential, side effects and variable tolerance levels between human and animal subjects (McBride et al., 2007) . The novel 4-AP derivative, 4-aminopyridine-3-methanol (4-AP-3-MeOH), has emerged as a potential more potent, better tolerated and effective alternative. 4-AP-3-MeOH was shown to significantly augment axonal conduction and action potential amplitude following acute SCI (Page et al., 2018; Y et al., 2014) and restore conduction in *ex vivo* preparations of multiple sclerosis (MS) (Leung et al., 2011). Therefore, 4-AP-3-MeOH could potentially complement eEMC therapy.

Experimental goal and hypothesis

The extent to which potassium channel blockers enable upper limb movement in combination with spinal stimulation and motor training has not yet been investigated. Therefore, the present study seeks to determine the effects of potassium channel ion blockers, 4-AP and 4-AP-3-MeOH on the activation of spared networks and functional recovery of upper limbs following incomplete cervical SCI. We hypothesize that animals that receive eEMC and motor training supplemented with potassium channel blockers, 4-AP and 4-AP-3-MeOH will demonstrate improved forelimb control and strengthened muscle responses compared to animals that receive only training and eEMC. We expect that 4-AP-3-MeOH will result in greater muscle and conduction responses and functional improvements compared to 4-AP at the same dosage.

MATERIALS AND METHODS

Animal training

All procedures were performed according to institutional and governmental regulations and in accordance with the guidelines established and delineated by the UCLA Chancellor's Animal Research Committee (ARC) concerning the ethical use of animals. Female Long-Evans rats (250-300 g; n = 9) were housed individually at a constant 25 C, 40% humidity and maintained on a 12-hour light/dark cycle. Rats were food deprived to 80-90% of their original weight and given supplemental feeding daily to maintain their weight.

Reaching and grasping training and testing

Prior to reaching and grasping training, animals were acclimated to the testing environment. Rats were placed individually inside a translucent acrylic testing box (18 cm x 15 cm x 31 cm) with a small opening in the front (3 cm x 1.5 cm). During reaching and grasping training, a sugar pellet (45 mg, Dustless Precision Pellets, Bio-Serv, Frenchtown, NH, USA) was placed on a platform 1 cm from the front opening. Animals were trained daily for 6 weeks. Rats were presented with 20 total pellets during every training and testing sessions and were closely monitored for their preferred paw. The success rate was calculated taking the ratio of the total number of pellets eaten to the total number of reaching/grasping attempts. Baseline success rates, EMG and sMEP recordings were obtained pre-injury and motor function was measured every week for 4 weeks after injury.

Surgical Procedures

Under aseptic conditions, rats were deeply anesthetized with isoflurane gas (1.5-2.5%) administered via facemask. Throughout surgery, body temperature was maintained at 37° C using a heating pad to prevent hypothermia. The depth of anesthesia was assessed prior to and regularly throughout surgery using a toe pinch.

Rats were given an analgesic (Buprenorphine HCl, 0.03 mg/kg s.c) and an NSAID (Rimadyl, 5 mg/kg s.c.) every 12 hours for 2 days post-surgery and an antibiotic (Enrofloxacin, 10 mg/kg s.c.) every 12 hours for 5 days post-surgery. Additionally, animals were given *ad libitum* access to food one week post-surgery to allow animals to fully recover.

Surgery I: EMG recording electrodes and spinal stimulator implants

To implant the transcranial stimulator, a skin incision was made along the midline of the skull and the overlying tissue was reflected laterally. The skull was dried, and screws were affixed firmly into the bone. An amphenol–recording unit (Omnetics, Minneapolis, MN, USA) was placed between the screws and secured to the skull with dental cement. Intramuscular EMG recording electrodes were implanted unilaterally in the preferred paw. The deltoid, biceps brachii, pronator teres, flexor digitorum, and extensor digitorum were selected for their relevance to reaching and grasping tasks.

To implant the spinal stimulator, a partial laminectomy was performed at C6 and T1 vertebral levels to expose the C6 and C8 spinal cord levels. Teflon-coated stainless-

steel wires (AS632, Cooner Wire, Chatsworth, CA, USA) from the amphenol–recording unit was passed subcutaneously to the laminectomy sites. Stimulation electrodes were made by removing a 1 mm of the Teflon to expose the stainless-steel wire on the surface facing the spinal cord. The electrodes were sutured to the dura at the midline of spinal levels C6 and C8. A common reference wire was created by stripping 1 cm of Teflon from the distal end and inserted subcutaneously near the shoulder of the dominant paw.

Surgery II: Cervical spinal cord injury

To induce an incomplete cervical SCI, a longitudinal midline skin incision was made dorsal to the spinal column and the underlying back muscles were reflected laterally. A partial laminectomy was performed at C3 and C4 vertebral levels to expose the spinal cord. The C4 dorsal funiculi was crushed bilaterally by placing the tips of fine forceps 2 mm apart, inserting the tips 2 mm in depth into the spinal cord, and then squeezing the tips together and holding them closed for 20 seconds. The injury crushes the dorsal funiculus and damages the corticospinal tract to impair fine motor movements but allow gross upper muscle movement. Histological evaluation of this injury has been reported previously.

4-AP and 4-AP-Me-3-OH Preparation and Administration

4-AP (Sigma Aldrich) was dissolved in sterile saline and delivered intraperitoneally as a bolus injection (0.32 mg/kg) one hour prior to reaching and grasping training or testing to achieve peak blood plasma levels. 4-AP was administered every other day 2-, and 3-

weeks post-SCI. The dosage and bolus delivery method are optimal for maintaining clinically meaningful plasma levels of 4-AP (Sindhurakar et al., 2017). 4-AP-Me-3-OH (Fischer Scientific) was similarly prepared and administered during 4- and 5-weeks post-SCI.

Epidural spinal stimulation and spinal motor-evoked potential recordings

Bipolar (C6- C8+ and C6+ C8-) epidural electrical stimulation pulses (200 µsec pulse width) were delivered at 2 Hz at the minimum current needed to generate a spinally evoked potential (sMEP) in the forelimb muscles (Grass SIU5; Grass Instruments, Warwick, RI, USA). The signals were filtered (band-pass; 30-1000 Hz) and amplified (1000x) using a multichannel analog amplifier (Differential AC amplifier Model 1700, AM-Systems Inc., Sequim, WA, USA). The amplified signals were digitized at 10 KHz and recorded using PowerLab and LabChart Pro acquisition and analysis system (AD Instrument, New Zealand). Roughly 20 pulses and evoked potentials were recorded during non-active periods.

During reaching and grasping tasks, animals were epidurally stimulated by delivering subthreshold monophasic stimulation pulses to enable, but not initiate forelimb motor movements. Subthreshold was defined as 95% of the current threshold necessary to generate a palpable muscle twitch in the implanted forelimb muscles. Animals were epidurally stimulated during every training session. The beginning of each reaching and grasping cycle was recorded in real-time using the Video Capture Module of LabChart Pro in order to identify relevant EMG signals. During testing, success rates, muscle

activation and sMEPs were collected pre- (eEMC only) and post-treatment with (eEMC and 4-AP or 4-AP-3-MeOH).

Data analyses

The raw EMG recordings were rectified to analyze the area under the curve and calculate the integrated EMG. Post-treatment values were normalized to pre-treatment values. Reaching and grasping success rate was calculated by taking the percent ratio of successful attempts to total attempts. To analyze sMEP, the latency to muscle activation was determined by measuring the time to reach peak amplitude after the stimulation pulse. The latency period was divided into early activation (10 ms after stimulation) and late activation (10-30 ms after stimulation). Latency to peak amplitude was calculated for 10 pulses for each muscle.

Immunohistochemistry

A lethal injection of sodium pentobarbital (100 mg/kg body weight) was administered intraperitoneally prior to transcardial perfusion with 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffered Saline (PBS). Prior to and throughout the perfusion, the depth of anesthesia was assessed using the toe-pinch reflex. The cervical spinal cord was carefully dissected and post-fixed overnight in 4% PFA at 4°C and cryoprotected in 30% sucrose in PBS. Tissue segments containing spinal levels C6-C8 were embedded in Neg-50™ (Richard-Allan Scientific) and stored for future histology and immunohistochemical analysis.

Statistics

All data are reported as the standard error of the mean. A two-tailed paired or unpaired t-test was used to difference the differences in the success rates, sMEPs and EMG pre- and post-pharmacological treatment and pre- vs. post-injury. Repeated measures of analysis of variance (ANOVA) was used to evaluate overall differences. Individual group differences were asses using Bonferroni post-hoc test. Statistical analysis was performed using Prism 8 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant if $p < 0.05$ (*) and highly significant if $p<0.0001$ (***).

RESULTS

Treatment with eEMC and potassium channel blocker, 4-aminopyridine (4-AP), acutely restores reaching and grasping function and increases forelimb muscle EMG after cervical spinal cord injury

EMG activity was measured in the five forelimb muscles during reaching and grasping tasks before and after cervical SCI. At 1-week post injury, animals received their first doses of 4-AP. Responses were measured pre- (eEMC only) and one-hour post- 4-AP administration (eEMC + 4AP) when 4-AP reached peak blood plasma levels. Pre-injury EMG amplitudes were increased compared to baseline. The first 4-AP treatment did not increase EMG activity across all muscle groups (deltoid: 0.286 ± 0.0033 vs. 0.51 ± 0.010; bicep: 0.192 ± 0.072 vs. 0.058 ± 0.012; pronator: 0.293 ± 0.061 vs. 0.168 ± 0.019; flexor: 0.133 ± 0.020 vs. 0.037 ± 0.013; extensor: 1.148 ± 0.201 vs. 0.294 ± 0.032) (Figure 3B) and we observed minimal improvement in reaching and grasping

success rates (0.24 ± 0129 vs. 0.30 ± 0.128) (Figure 2A). Post-treatment EMG burst activity was also consistently low compared to baseline levels in all muscle groups, except the extensor which demonstrated a brief burst in activity (Figure 3A).

Following a week of reaching and grasping training, success rates improved after treatment with 4-AP compared to the previous week (0.30 ± 0.128 vs. 0.67 ± 0.127) (Figure 2A). Following treatment with 4-AP, success rates nearly doubled compared to pre-treatment values (0.35 ± 0.120 vs. 0.67 ± 0.127) (Figure 3A). The improvement in success rates were accompanied by increases in EMG activity in all muscles (deltoid: 0.31 ± 0.044 vs. 0.37 ± 0.027; bicep: 0.07 ± 0.014 vs 0.10 ± 0.011; pronator: 0.08 ± 0.015 vs. 0.12 ± 0.005; flexor: 0.13 ± 0.016 vs. 0.17 ± 0.006; extensor: 0.06 ± 0.013 vs. 0.09 ± 0.003) (Figure 3C). 4-AP significantly increased activation in the deltoid, flexor and extensor muscles ($p<0.0001$, paired t-test), as well as the pronator ($p<0.0043$, paired t-test) (Figure 3C).

After two weeks of treatment (3 weeks post-SCI), the upward trend in reaching and grasping success rates persisted both pre- and post- treatment with 4-AP. Pre-treatment values were nearly equal to the post-treatment values the week prior (0.67 ± 0.089 vs. 0.66 ± 0.127). After treatment with 4-AP, success rates increased to near baseline values (0.79 ± 0.045 vs. 0.79 ± 0.00, p-value = n.s.) (Figure 2C). At this time, EMG activity was increased post-treatment compared to pre-treatment across all muscle groups (deltoid: 0.26 ± 0.03 vs. 0.32 ± 0.06; bicep: 0.174 ± 0.018 vs 0.230 ± 0.028; pronator: 0.082 ± 0.011 vs. 0.105 ± 0.020; flexor: 0.105 ± 0.012 vs. 0.456 ±

0.068; extensor: 0.071 ± 0.012 vs. 0.066 ± 0.011) (Figure 3D). Muscle activation was now significantly increased in the bicep (p-value <0.05, paired t-test), which was the only muscle group that was not substantially activated following 4-AP treatment the week prior. The pronator and flexor muscles continued to experience highly significant increases (p-value<0.0001, paired t-test) in EMG activity in response to 4-AP.

In response to the recovery of reaching and grasping ability, muscle responses at this timepoint were normalized and compared to pre-injury baseline values. Pre- and post-treatment EMG activity was increased compared to baseline in the deltoid, bicep and flexor muscles (Figure 3E). The response was consistent among these three muscles, where post-treatment values were increased compared to pre-treatment values. The flexor muscle demonstrated a near 5-fold increase in EMG activation following treatment with 4-AP. A sharp post-treatment response was not observed in any other muscle group at any other timepoint. The extensor showed decreased muscle activation pre- and post-treatment compared to pre-injury values (Figure 3E). Post-treatment EMG values in the pronator showed muscle activation return to near baseline values (0.105 ± 0.020 vs. 0.104 ± 0.011) (Figure 3F).

Novel 4-AP derivative, 4-aminopyridine-3-methanol (4-AP-3-MeOH) does not lead to functional forelimb recovery or EMG muscle activation

Following the functional recovery plateau after two weeks of treatment with 4-AP, we treated animals with the novel 4-AP derivative, 4-aminopyridine-3-methanol (4-AP-3-MeOH) which reportedly improves axonal conduction at much lower doses than 4-AP.

Animals received the first dose of 4-AP-3-MeOH at 4 weeks post-SCI. Animals received equivalent doses of 4-AP-3-MeOH as 4-AP and underwent the same reaching and grasping training and spinal stimulation protocols. Reaching and grasping success rates did not change during initial pre- and post-treatment of 4-AP-3-MeOH (0.69 ± 0.057 vs. 0.625 ± 0.048) (Figure 2B). EMG amplitudes slightly decreased after drug administration, similar to what was observed during the first administration of 4-AP (deltoid: 0.359 ± 0.049 vs. 0.242 ± 0.021; bicep: 0.237 ± 0.037 vs. 0.134 ± 0.015; pronator: 0.124 ± 0.017 vs. 0.091 ± 0.014; flexor: 0.140 ± 0.015 vs. 0.117 ± 0.015; extensor: 0.140 ± 0.015 vs. 0.074 ± 0.013) (Figure 4A).

After a week of treatment (5 weeks post-SCI), pre-treatment success rates were increased compared to the post-treatment values of the previous week (0.70 ± 0.130 vs. 0.625 ± 0.048) (Figure 2B). Following treatment with 4-AP-3-MeOH, success rates decreased (0.70 ± 0.130 vs. 0.562 ± 0.156) (Figure 2A and 2B) to the lowest values since starting treatment with 4-AP-3-MeOH. Additionally, EMG continued to decrease post-treatment across all muscle groups (deltoid: 0.305 ± 0.030 vs. 0.245 ± 0.021; bicep: 0.185 ± 0.022 vs. 0.121 ± 0.015; pronator: 0.122 ± 0.015 vs. 0.082 ± 0.010; flexor: 0.131 ± 0.014 vs. 0.102 ± 0.011; extensor: 0.101 ± 0.014 vs. 0.062 ± 0.010) (Figure 4B).

4-AP and 4-AP-3-MeOH decrease latency to spinally evoked peak amplitudes in forelimb muscles

Spinal motor-evoked potentials (sMEPs) were delivered through the implanted epidural stimulator from the spinal cord to the forelimb muscles. Roughly 20 pulses were recorded during non-active periods of activity before and after pharmacological treatment.

The time to peak amplitude increased immediately after injury, where amplitudes for all muscles peaked within the 20-30 ms range (late activation) after stimulation. Initial treatment with 4-AP did not have an effect on latency times (1 week post-SCI) (not shown). After one week of treatment with 4-AP (2 weeks post-SCI), latency decreased in all muscles before and after treatment, in all muscles except the deltoid (deltoid: 9.64 ± 1.693 vs. 10.782 ± 1.103; bicep: 12.291 ± 0.1.449 vs. 06.791 ± 0.818; pronator: 8.712 ± 1.424 vs. 8.062 ± 1.225; flexor: 11.98 ± 3.129 vs. 5.002 ± 0.709; extensor: 11.997 ± 1.145 vs. 7.284 ± 0.819) (Figure 5A). Values for the bicep, flexor (p-value<0.05, Mann-Whitney test) and extensor (p-value<0.0001, Mann-Whitney test) significantly decreased and values shifted from late to early activation (Figure 5A). After an additional week of 4-AP treatment (4 weeks post-SCI), latency time decreased before and after treatment in the deltoid and continued to decrease in all muscles, except the pronator (deltoid: 9.295 ± 1.31 vs. 7.89 ± 1.056; bicep: 5.229 ± 0.928 vs. 4.055 ± 0.154; pronator: 5.81 ± 0.559 vs. 5.835 ± 0.792; flexor: 4.738 ± 0.658 vs. 4.002 ± 0.246; extensor: 6.057 ± 0.662 vs 4.865 ± 0.41) (Figure 5B). Nearly all pre- and post- treatment amplitudes now peaked closer to the 1-10 ms range (early activation). The post-

treatment latency times decreased between one and two weeks of treatment and were significantly lower in the bicep ($p<0.0001$, Mann-Whitney test).

Similar measurements were performed after treating animals with 4-AP-3-MeOH. Following the first week of treatment (5 weeks post-SCI) time to peak decreased pre- and post-treatment in the flexor and extensor muscles (deltoid: 9.636 ± 1.041 vs. 10.216 ± 1.292; bicep: 4.821 ± 0.63 vs. 7.671 ± 1.546; pronator: 7.671 ± 1.546 vs. 3.892 ± 0.224; flexor: 5.443 ± 1.102 vs. 4.432 ± 0.507; extensor: 8.068 ± 1.172 vs. 6.3 ± 0.795) (Figure 5C). After an additional week of treatment with 4-AP-3-MeOH, time to peak decreased pre- and post- treatment in all muscle groups (deltoid: 7.83 ± 1.222 vs. 6.67 ± 0.932; bicep: 8.443 ± 2.008 vs. 4.513 ± 0,482; pronator: 7.01 ± 1.556 vs. 4.337 ± 0.42; flexor: 5.79 ± 1.255 vs. 3.86 ± 0.413; extensor: 9.457 ± 1.208 vs. 6.42 ± 0.763) (Figure 5D). The extensor muscles (p-value<0.0001, Mann-Whitney test) demonstrated significant decreases in latency times. Post-treatment latency times decreased in all muscles between one and two weeks of treatment with 4-AP-3-MeOH. By the end of the second week of treatment, all pre- and post- amplitudes peaked between 0-10 ms (early activation).

Animals were trained and treated with 4-AP and 4-AP-3-MeOH for the same amount of time. In order to evaluate the effectiveness of one treatment against the other, the time to peak was compared at equivalent time points after one week of treatment with the (Week 2 vs. Week 4) (Figure 5E) and after two weeks of treatment (Week 3 vs. Week 5) (Figure 5F). After one week of treatment, 4-AP-3-MeOH resulted in lower latency times

compared to 4-AP in all muscle groups, and values were significantly decreased in the bicep and pronator muscles (p-value<0.05, Mann-Whitney test). Similarly, after two weeks of treatment, 4-AP-3-MeOH lead to lower latency times in all muscles, except the extensor (4.865 ± 0.41 vs. 6.42 ± 0.763). These values were relative to the activation window (0-30 ms) rather than the pre-treatment values. The time to reach peak was significantly decreased following treatment with 4-AP-3-MeOH compared to 4-AP in the pronator and flexor muscles (p-value<0.05, Mann-Whitney test).

DISCUSSION

Previously, we demonstrated that it is possible to modulate cervical spinal networks through subthreshold stimulation of spared networks. eEMC combined with pharmacological activation increases baseline excitability of relevant motor units and supports the initiation of voluntary movement. Additionally, promising studies have shown that spinal stimulation can similarly restore motor function in the upper limbs by increasing grip strength and fine motor movements in spinalized animals and human patients (Alam et al., 2018; Gad et al., 2018). The present study evaluated the acute effects of potassium ion channel blockers, 4-AP and 4-AP -3-MeOH on forelimb muscle activation and reaching and grasping movements in combination with subthreshold spinal stimulation over C6 and C8 after incomplete cervical SCI.

We compared motor performance supplemented with eEMC before and after treatment with each potassium channel blocker for two weeks. Initial treatment with both drugs did not demonstrate immediate improvements in motor function and muscle activation.

However, after one week of treatment with eEMC and 4-AP, animals showed improve reaching and grasping ability and greater muscle activation that persisted to the next week. Reaching and grasping ability returned to pre-injury levels. Muscle activation was greater compared to pre-injury values in the deltoid and bicep and flexor muscles but decreased in the extensor muscles. Pronator muscle strength returned to near pre-injury values.

The EMG data show increased muscle activity during motor tasks immediately after injury. This hyperactivity is a hallmark response after injury. After the first injection of 4-AP, we observed a significant decrease in muscle activation, suggesting 4-AP may function by modulating hyperexcitability. Normally, 4-AP functions to increase hypoexcitability by reducing potassium efflux. The mechanism of action of 4-AP is not well established in SCI, but it has been documented to act on calcium channels and effect synaptic release At this timepoint, we did not observe measurable improvement in reaching and grasping ability, which could be attributed to lack of training and exposure to the testing environment for a week.

Continued treatment with 4-AP shows persistence of muscle response and function. We observed consistent improvement in motor function after one and two weeks of treatment. The motor improvement was now accompanied by increased muscle activation across all groups. Additionally, 4-AP improved conduction. All muscles demonstrated earlier activation times, with all muscles reaching peak amplitude with the early activation window after two weeks of treatment. These results are expected given

the mechanism of action of 4-AP, but extremely significant in that they affect activation as well as function of the arm and hand muscle. Currently, 4-AP is clinically approved to treat locomotor dysfunction and gait abnormalities only. Here we have shown that 4-AP can significantly improve motor function in the upper limbs and return pre-injury function in the arm and hand.

We suggest that continued and regular use of 4-AP is necessary for motor improvement. Much like eEMC and motor tasks, it is likely that 4-AP helps ‘train’ the spared circuits over time by enabling damaged circuits and augmenting spinal stimulation. Continued combination treatment with 4-AP could have allowed eEMC and training to better target and modify relevant circuits for reaching and grasping. The effects of 4-AP persist, but based on the reduction in performance when switching to 4-AP-3-MeOH, they do not last for more than two days after two weeks of treatment. Additionally, majority of demyelination after SCI occurs at 14 days post-injury. These results suggest that 4-AP can be used neuroprotectively at crucial timepoints of myelin damage, though further immunohistological data is needed to assess the extent of demyelination following 4-AP treatment.

While 4-AP has led to improved clinical outcomes, several studies have claimed that the novel derivative, 4-AP-3-MeOH is more potent and more effective in the restoration of axonal conduction. Here, we evaluated the two potassium channel blockers against one another in order to substantiate these claims and determine whether one blocker better complemented eEMC treatment and lead to improved functional recovery.

We found that 4-AP-3-MeOH (0.32 mg/kg) did not significantly augment EMG activity in the deltoid, biceps, pronator, extensor or flexor muscles when compared to 4-AP (0.32 mg/kg). With continued use, 4-AP-3-MeOH appeared to decrease muscle activation. Animals displayed reduced ability to perform reaching and grasping tasks when treated with 4-AP-3-MeOH versus 4-AP. However, 4-AP-3-MeOH increased axonal conduction by decreasing the latency to muscle activation in the bicep, pronator and flexor muscles. Previous studies suggest that 4-AP-3-MeOH affects axonal circuits differently than 4-AP in that 4-AP-3-MeOH does not change the overall electrical response of axons to multiple stimuli. Based on these findings, we suggest that in order for potassium channel blockers to work synergistically in eEMC SCI treatment, they should allow circuits to flexibly respond to various stimuli (motor, sensory, proprioceptive) during recovery from SCI in order to complement eEMC treatment.

Though 4-AP-3-MeOH has been found to be more effective than 4-AP at greater dosages (1 and 5 mg/kg) (Page et al., 2018), previous studies have not considered translational applications of those dosages. Here, we used 0.32 mg/kg of both potassium channel blockers – a dosage that leads to peak blood plasma levels in rodents similar to that found in human patients taking the commercially available form of 4-AP, Ampyra (Acorda Therapeutics, Inc.). Therefore, further research is needed to establish 4-AP-3-MeOH as a viable alternative to 4-AP in *in vivo* applications and functional recovery. While 4-AP-3-MeOH can lead to greater axonal conduction *ex vivo* and can be used to restore axonal conduction in following cervical lesions, 4-AP is a

more effective in the restoration of upper motor function and a better candidate in clinical applications for SCI.

4-AP may have appeared to have a stronger complementary effect than 4-AP-3-MeOH due to the timing of the treatments. The same groups of animals were tested with 4-AP until recovery plateau and then 4-AP-3-MeOH. It is possible that 4-AP-3-MeOH resulted in limited improvement compared to 4-AP because animals had already received 2 weeks of treatment with 4-AP. However, our data suggests that 4-AP needs to be used consistently, was eliminated before treatment with 4-AP-3-MeOH. It is also unlikely extensive neuroplastic reorganization occurred in such a short time span. Therefore, we can still consider the role of 4-AP-3-MeOH in combination therapy. We also compared the same number of treatments and dosages with each drug, not necessarily the same injury severity. Nonetheless, we evaluated 4-AP-3-MeOH as potential SCI treatment, and while it shows efficacy in conduction velocity, further research is needed to fully evaluate its potential as a pharmacological agent in combination SCI treatment.

Conclusions

Acute administration of 4-AP complemented eEMC and motor training treatment. 4-AP effectively restores upper limb motor function by increasing muscle excitability and activation. 4-AP-3-MeOH derivative similarly restores muscle excitability but appears to overly inhibit muscle activation and fails to restore functional reaching and grasping movements following acute administration. The use of 4-AP in SCI has been widely inconclusive, but here we show that 1) 4-AP has broad therapeutic effects and can be

used to restore upper limb function and responsivity, 2) 4-AP synergistically works with eEMC and motor training to enable spared spinal circuitry following SCI. Currently, 4-AP is FDA approved for use in MS and is well-tolerated by humans, affordable and accessible Therefore, 4-AP could be integrated into clinical eEMC therapies and dramatically bring us closer to a cure for paralysis. Out findings promote clinical support of 4-AP in combination with, eEMC and training to restore hand and arm function following traumatic cervical SCI.

FIGURES

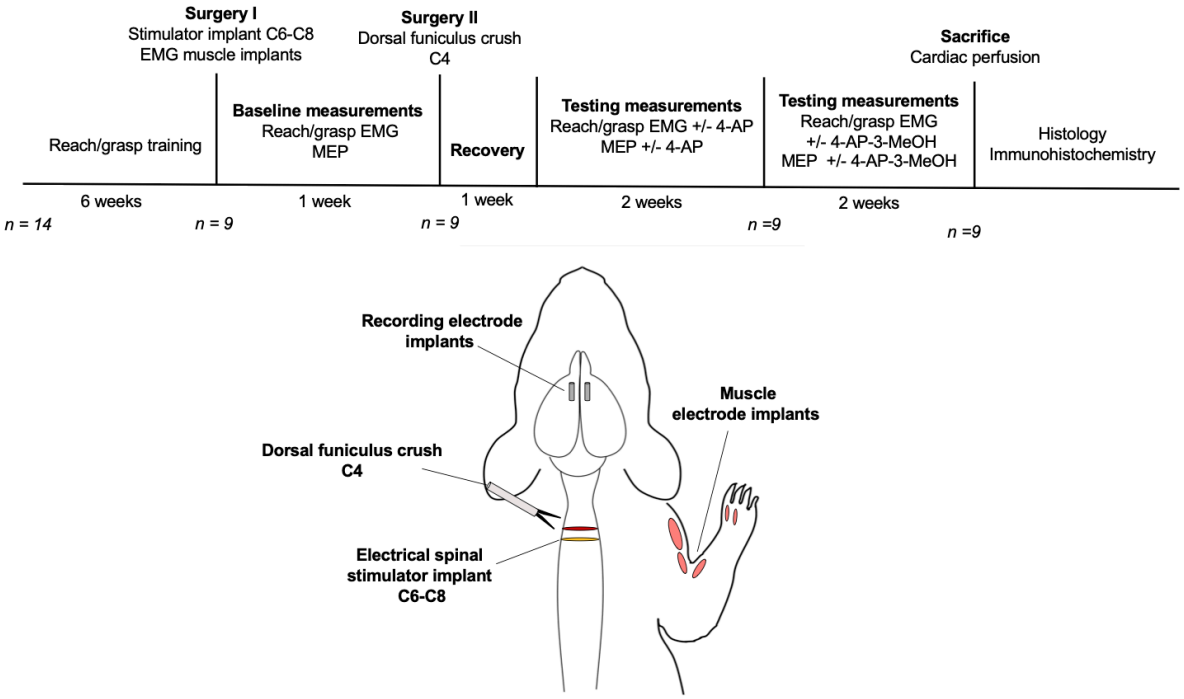

Figure 1: Experimental design and timeline
Nine rats were trained to reach and grasp sugar pellets and monitored for their preferred paw. Animals that demonstrated mastery of the task and established paw dominance were implanted with EMG recording electrodes in the forelimb muscles of the dominant hand and epidural stimulating electrodes over spinal levels C6 and C8 (Surgery I). One week after implant surgery, the following baseline measurements were recorded: reaching and grasping performance, reaching and grasping EMG and motor-evoked potentials (MEP). Afterwards, all animals received spinal cord lesions by carefully crushing the dorsal funiculus of the C4 spinal level (Surgery II). Animals were trained and treated with 4-AP every other day and tested weekly for 3 weeks post-lesion. During testing, reaching and grasping performance, EMG, and MEPs were recorded before and after administration of 4-AP. Animals were epidurally stimulated at subthreshold during EMG recordings and stimulated at 20 and 40 Hz to induce MEPs. Following recovery plateau with 4-AP, animals were similarly treated with the drug derivative, 4-aminopyridine-3-methanol (4-AP-3-MeOH) for 2 weeks and tested for motor ability and function.

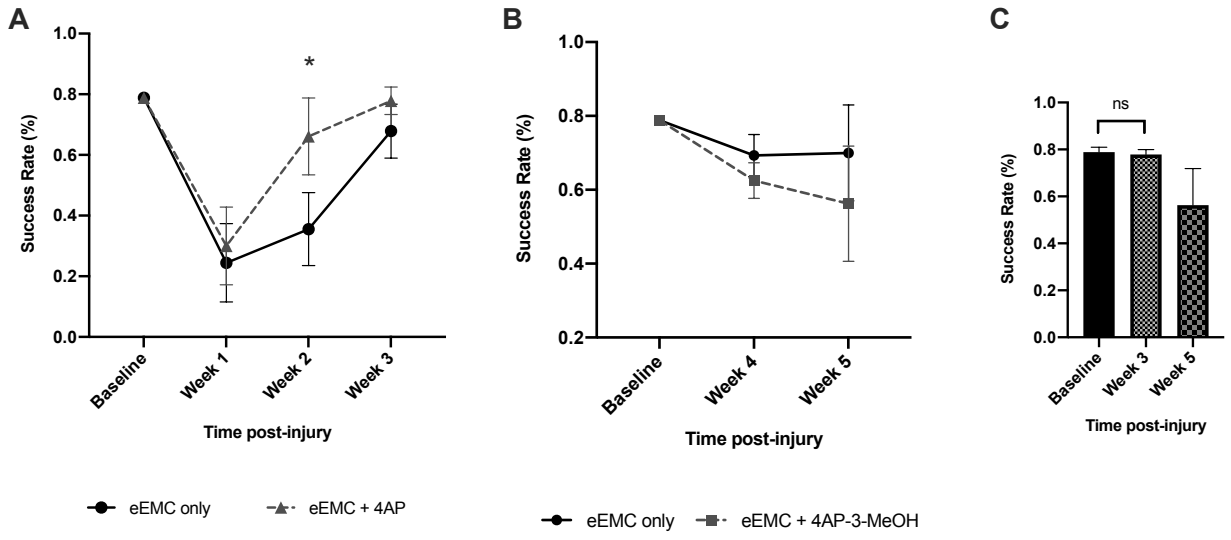

Figure 2: Treatment with eEMC and potassium channel blocker, 4-AP acutely restores reaching and grasping function after cervical spinal cord injury
(A. Mean (±SEM) reaching and grasping success rates at baseline, 1, 2, and 3 weeks post-lesion before (solid black line) and one hour after administration of 4-AP (grey dotted line). Animals received first dose of 4-AP at week 1 timepoint (p-value 0.3466) and were treated every other day thereafter. After a week of treatment (Week 2), animals demonstrated minimal improvement (24.4% to 35.6%) in pre-treatment reaching and grasping ability. After receiving 4-AP, reaching and grasping function significantly improved. (35.6% to 66.1%, **p = 0.01683). After two weeks of treatment, animals showed improved reaching and grasping ability prior to 4-AP treatment compared to the previous week (67.9% from 35.6%). Success rate increased after 4-AP treatment, but was not significantly increase compared to pre-treatment levels that week.
(B) Mean (±SEM) reaching and grasping success rates at 4 and 5 weeks post-lesion before (solid black) and one hour after administration of 4-AP-3-MeOH (blue line). Animals received first dose of 4-AP-3-MeOH at the week 4 timepoint. Animals received equivalent doses of 4-AP-3-MeOH and underwent the same training and stimulation protocols as the first drug. Reaching and grasping function did not change before and after drug administration (69% to 70%). After one week of treatment, animals showed a slight decrease in success rate before (69% to 62.5%) and after (70% to 56.25%) eEMC and 4-AP-3-MeOH treatment.
(C) Comparison of baseline to mean (±SEM) reaching and grasping success rate after two weeks of 4-AP treatment and 4-AP-3-MeOH. Animals were able to regain reaching and grasping ability to near pre-injury levels (77.9% compared to 78.8%) when treated with eEMC and 4-AP but did not demonstrate the same motor improvement when treated with eEMC and 4-AP-3-MeOH.

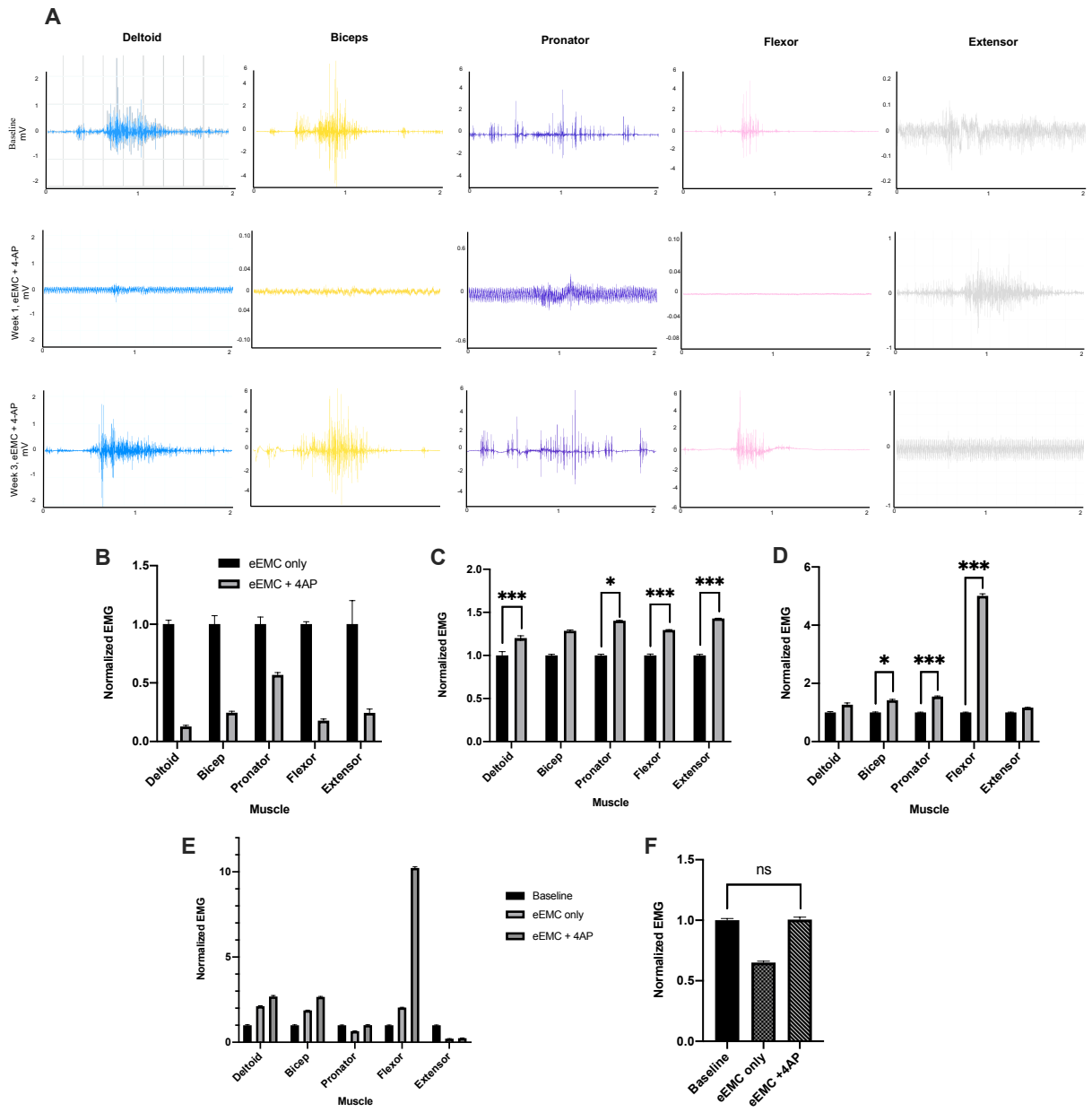

Figure 3: Treatment with eEMC and potassium channel blocker, 4-AP increases forelimb muscle EMG after cervical spinal cord injury

(A) Raw EMG signals from the forelimb muscles during reaching and grasping at baseline, 1, and 3 weeks post-injury.

(B) Comparison of the mean (±SEM) integrated EMG values (n = 20 reaching and grasping trials, 9 animals) before and after initial administration of 4-AP (1 week post-injury). Treatment with 4-AP decreased EMG activity in all forelimb muscles.

(C) Comparison of the mean (±SEM) integrated EMG values before and after administration of 4-AP after one week of treatment (2 weeks post-injury). Treatment with 4-AP significantly increased EMG activity in all forelimb muscles, but the biceps (p-value > 0.05).

(D) Comparison of the mean (±SEM) integrated EMG values before and after administration of 4-AP after two weeks of treatment (3 weeks post-injury). This time, treatment with 4-AP significantly increased EMG activity in the biceps. EMG activity remained significantly increased in the pronator after treatment with 4-AP. EMG activity was not significantly increased in the deltoid or extensor. EMG increased 5-fold after 4-AP administration in the flexor muscle.
(E) EMG values after two weeks of treatment with 4-AP normalized to pre-injury baseline levels. Animals regained functional motor ability at this timepoint. 4-AP appeared to increase muscle activation compared to baseline in all muscles, except the extensor.
(F) Mean (±SEM) integrated EMG values pre- and post-treatment with 4-AP in the pronator muscle after two weeks of treatment. When treated with a combination of eEMC and 4-AP, EMG values returned to pre-injury baseline values.

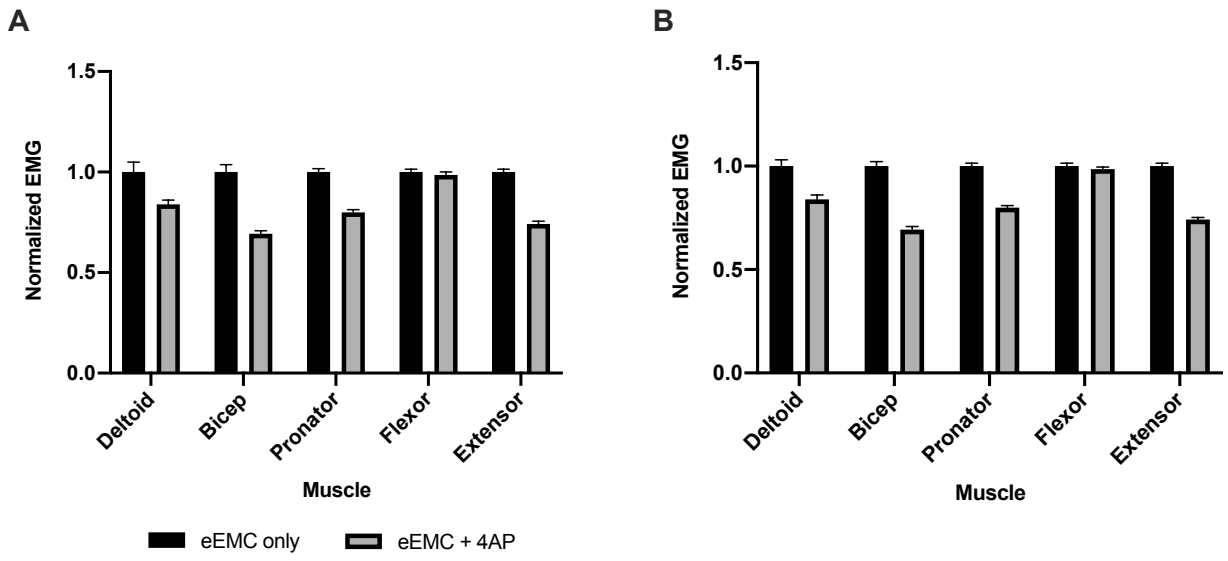

Figure 4: 4-AP-3-MeOH decreases forelimb EMG muscle activation after cervical spinal cord injury

(A) Comparison of the mean (±SEM) integrated EMG values before and after treatment with 4-AP-3-MeOH after one week of treatment (4 weeks post-injury). EMG activity decreased in all muscles.

(B) Comparison of the mean (±SEM) integrated EMG values before and after treatment with 4-AP-3-MeOH after two weeks of treatment (5 weeks post-injury). EMG activity decreased in all muscles.

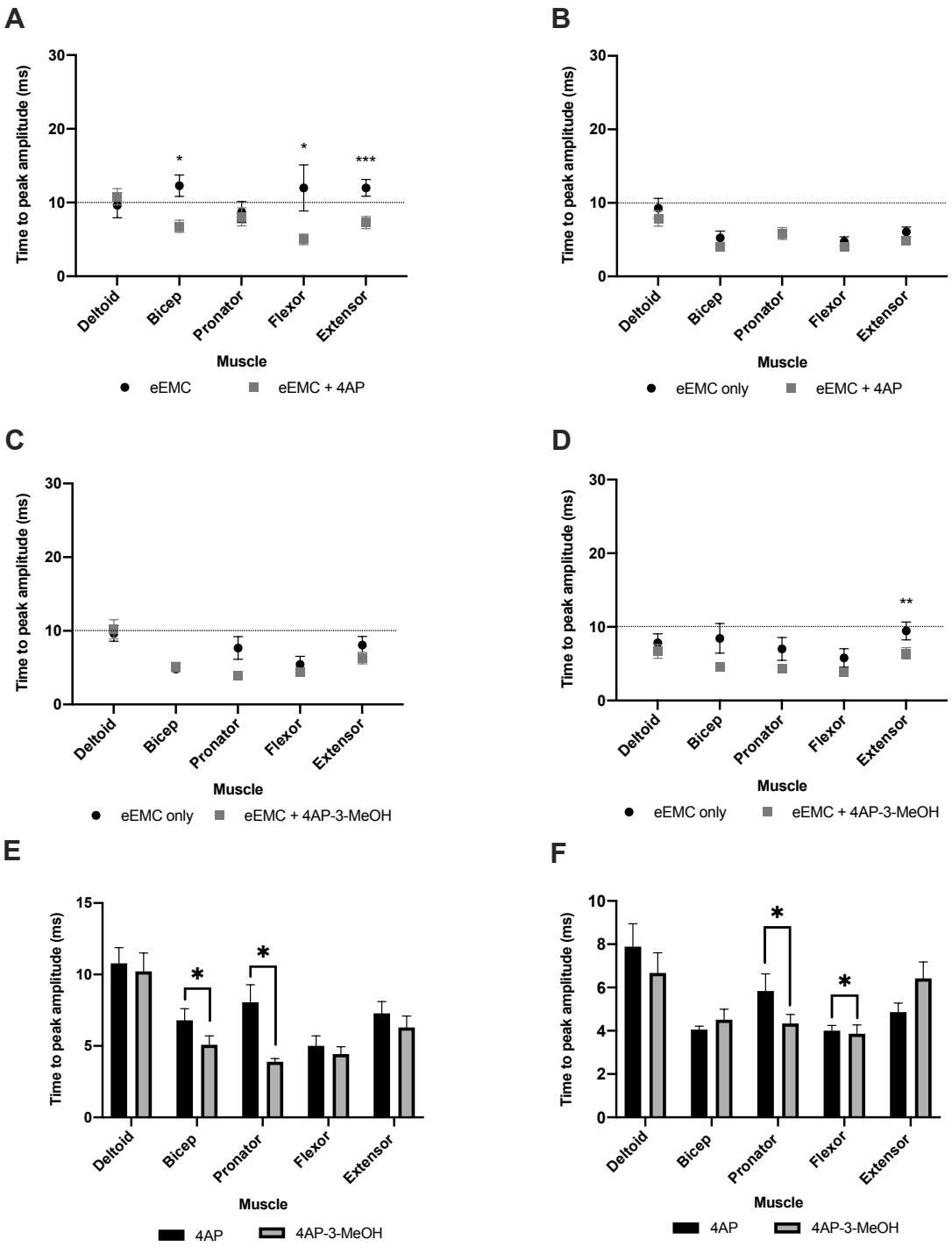

Figure 5: 4-AP and 4-AP-3-MeOH decrease times to peak amplitudes following spinally evoked potentials

(A) Time to reach peak EMG amplitude (n = ~15 pulses, 4 animals) before and after treatment with 4-AP after one week of treatment (2 weeks post-injury). Dotted line separates early activation phase (0-10 ms) from late activation phase (20-30 ms). 4-AP

significantly decreased latency in the bicep, flexor and extensor and EMG reached peak amplitude in the early activation phase.
(B) Time to reach peak EMG amplitude before and after treatment with 4-AP after two weeks of treatment (3 weeks post-injury). 4-AP continued to decrease latency in all muscles, except the pronator. All muscles reached peak amplitude within the early activation phase.
(C)Time to reach peak EMG amplitude before and after treatment with 4-AP-3-MeOH after one week of treatment (4 weeks post-injury). All muscles experienced early activation prior to treatment. 4-AP-3-MeOH further decreased latency and increased early activation.
(D) Time to reach peak EMG amplitude before and after treatment with 4-AP-3-MeOH after two weeks of treatment (5 weeks post-injury). Activation was significantly increased in pronator and flexor muscles.
(E) Comparison of peak latency between potassium channel blocking drugs after one week of treatment. 4-AP-3-MeOH significantly decreased peak latency compared to 4-AP in the bicep and flexor muscles. Other muscles similarly experienced earlier activation in response to 4-AP-3-MeOH compared to 4-AP.
(F) Comparison of peak latency between potassium channel blocking drugs after two weeks of treatment. Muscles showed different responses to 4-AP and 4-AP-3-MeOH. The pronator and flexor experienced significantly earlier activation when treated with 4-AP-3-MeOH while 4-AP decreased latency times in the bicep and extensor.

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